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14. ABSTRACT
The purpose of the current research is to determine whether the spray-on application of allogeneic keratinocytes in suspension will improve epidermal wound healing of vesicating burns induced by the chemical warfare agent sulfur mustard (HD). A beige SCID mouse model is used for these experiments which are being carried out in two phases. The first phase is dose ranging. The second phase tests the efficacy of spray keratinocytes (Universal Donor) at healing HD injuries. Dose ranging was carried out using HD (0, 80, 160, 320 µg) in methylene chloride delivered to the dorsum of depilated mice within an 8 mm diameter cloning ring. Under these conditions, the vehicle control caused patchy epidermal and follicular necrosis. As expected, the HD treatment generated confluent epidermal and follicular necrosis, endothelial cell necrosis, thrombi, and extravasation of fibrin and inflammatory infiltrate in the loose connective tissue. Induction of inflammation was independent of necrosis. Because of the confounding effects of methylene chloride, dose ranging and phase II experiments will be conducted with HD diluted in ethanol.

15. SUBJECT TERMS
Keratinocyte spray technology, sulfur mustard, Universal Donor

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Introduction

We had previously developed a cell based approach for treating acute cutaneous injuries and blistering disorders classified as epidermolysis bullosa (EB; Lin and Carter, 1992). The acute injury considered for therapeutic intervention is the result of exposure to sulfur mustard (bis-(2-chloroethyl) sulfide; HD), a chemical warfare agent that alkylates DNA, RNA and proteins. Prolonged cutaneous exposure to this agent results in vesication wherein small vesicles coalesce into large blisters that can take months to heal and require prolonged hospitalization (Papirmeister et al, 1991; Graham et al., 2005). Aggressive debridement of HD injuries that remove damaged cells and alkylated extracellular matrix ameliorates the sequlae and allows utilization of cell based therapies that either provide replacement tissue or promote healing with non-damaged host derived cells (Graham et al., 2005, 2006).

Under conditions of mass casualty or extensive injury (>40% body surface) availability of an off-the-shelf product which engrafts for prolonged times may be useful. In order to offer such a product, we had genetically engineered keratinocytes that were resistant to lymphocyte mediated cytotoxicity and which failed to stimulate proliferation of allogeneic lymphocytes even after growth with interferon–γ (Tafrov et al, 2004; Gao et al., 2006). This strain was produced by insertion of three sequences: shRNA against β2 microglobulin, antisense against the invariant chain (Ii) of MHC class II, and viral IL10 (Tafrov, Kalish and Simon, 2004). Suppression of β2 microglobulin and the invariant chain (Ii) of MHC class II was used to decrease antigen presentation. Viral IL10 was used to limit susceptibility to natural killer cells and to suppress production of proinflammatory mediators (Go et al, 1990; Vieir et al, 1991; Moore et al, 2001; Rousset et al, 1992). The strain of keratinocytes is the prototype of what we have termed the “Universal Donor” (UD) and was derived from normal human epidermal keratinocytes (strain SF). Both UD and SF keratinocytes can engraft onto a full-thickness wound made on beige SCID mice and generate a fully differentiated neo epidermis (Gao et al, 2006)

The purpose of the current research is to determine whether the spray-on application of allogeneic keratinocytes in suspension will improve epidermal wound healing of vesicating burns induced by the chemical warfare agent sulfur mustard (bis (2-chloroethyl) sulfide; HD). The experiment is being conducted in two Phases. Phase I is a dose ranging study to determine the dose regimen needed to induce a deep dermal/full thickness wound. Phase II examines the efficacy of spray keratinocytes at healing HD injuries. Both SF and UD cells will be used to treat HD injuries made on beige SCID mice with or without humanized immune systems.

Body

Due to delays in contracting, requirements to install and surety control the HD facility, and the initial limited availability of staff from the USAMRMC, the project start date was delayed until the beginning of May, 2008. At that time, the first dose ranging experiment was carried out. The amount of HD used was based on the observations in a mouse ear model of sulfur mustard injury demonstrating that a dose of 160 µg HD is sufficient to cause extensive cutaneous damage (Ricketts et al, 2000). In the current experiments animals were dosed with 50 µL methylene chloride (MC) alone (group 1-control, animal # 3-7), or dosed with 50 µL MC containing 320 µg HD (group 2; animal # 8-12), 160 µg HD (group 3; animal # 13-17), or 80 µg HD (group 4; animal #18-22) delivered in an 8 mm diameter cloning ring (see Figure 1A). It was necessary to keep the cloning ring for 5 min to ensure complete evaporation of the treatment. Beginning with animal #7, cloning ring rims were coated with Krytox® (Dupont, Wilmington, DE) to prevent leakage of the treatment that was observed with animals 3-6. All animals were kept for 24hr in the Class II B3 hood in the HD facility after which they were euthanized and skin samples taken for histopathology

and immunohistochemistry. Photographs were taken prior to euthanasia to document the noted edema and skin blanching (Table 1; Figure 1B). Edema was limited to the HD-treatment groups; these animals also showed blanching of the area. Blanching was also discernable in the MC control animals #4 and #7. Image analysis based upon blanching was conducted (Table 2).

It was noted that the 8 mm biopsies of sites treated either with MC alone or with MC containing different levels of HD tended to remain flat, whereas the biopsies of non-treated skin curled upon removal from the animal. This correlated with the histopathology, which showed muscle relaxation/fixation in the treated skin compared to non-treated skin (Figures 2D-F and 3A,B). Generally, skin absorption of MC is minimal due to its high evaporation rate and the adverse effects of MC are most often due to inhalation of the vapor. Certainly in the mouse ear model, MC evaporation was rapid (in the order of seconds rather than minutes). However, in the experimental setting used, evaporation required 2-5 minutes leading to the observed peripheral effects (see also Jucubovich et al, 2005).

Histopathologic evaluation revealed that treatment with MC generated patchy epidermal and follicular necrosis with variable loss of nuclear hematoxylin staining (Table 1, Figures 2, 4). In the HD treatment groups there was confluent epidermal and follicular necrosis with loss of hematoxylin staining in large areas up to the wound edge. In addition, in the HD treatment groups significant endothelial cell necrosis, thrombi, and extravasation of fibrin were noted (Figure 4D, E). Necrosis and loss of nuclear integrity was confirmed by staining for the high-mobility group box 1 (HMGB1) protein in epidermal keratinocytes and sebocytes (Figure 3). In keratinocytes as in many other cell types, HMBG1 serves as a DNA-binding protein that stabilizes nucleosomes and alters transcription. Its loss from the cell has been correlated with necrosis and induction of inflammatory responses (Scaffidi et al, 2002). However, in our experimental model system, although MC treatment reduced nuclear HMGB1 staining, the presence of inflammatory infiltrates was limited to the HD treatment group (Figure 4F, G). This observation is consistent with previous reports in mouse and human model systems which document release of other inflammatory cytokines and which demonstrate that HD promotes apoptosis (Ricketts et al, 2000; Arroyo et al, 2000, 1999; Ruff and Dillman, 2007; Rikimaru et al, 1991). The participation of HMGB1 later in the inflammatory process, perhaps secreted by other cells (i.e.: macrophage or dendritic cells) cannot be excluded from these results (Lotze and Tracey, 2005; Bonaldi et al, 2003). Within this data set, there was no correlation between skin blanching and HD injury or epidermal necrosis.

Because of the confounding effects of MC in this experimental setting, dose ranging will be conducted with HD diluted in ethanol. Baseline data (gross clinical observation, histopathology, and image analysis) on cutaneous ethanol effects will be obtained; significant adverse effects are not expected.

Key Research Accomplishments

- Set up a surety controlled HD facility
- Carried out first gene dosing experiments (Phase 1)
- Determined that in this experimental setting MC evaporation is limited leading to prolonged exposure and peripheral injury. Therefore, in the upcoming experiments ethanol will be used as the diluent, as adverse effects should be minimal. (Approvals from the Institutional Animal Care and Use Committee have been obtained.)

Reportable Outcomes

N/A

Conclusions

- Treatment with 50 µL MC with 2-5 minute exposure times causes peripheral injury with epidermal and follicular necrosis and involvement of muscle function.

- Preliminary results indicate that the presence of inflammatory infiltrate was limited to HD exposure demonstrating that inflammation was independent of necrosis. The release of other inflammatory cytokines is suggested (See also Ricketts et al, 2000; Arroyo et al, 1999).

- An alternative diluent for the HD is required in order to assess HD injury independent of the combined assault by MC; ethanol will be used for this purpose.

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Appendix 1. Methods

Dose Ranging:

The day prior to dose ranging the dorsum of each animal (Beige SCID: C.B.-17.B6-Prkdc^{scid}Lyst^{bg}/CRL, 7 weeks old; 20-24 grams) was shaved and depilated with Nair at the SBU animal facility; animals were lightly anesthetized with 2% isolfuorane for restraint. At that time each animal was numbered on the tail with an indelible marking pen. On the day of dose ranging the animals were placed in individual compartments of a cage that held 4 x 3 animals. Animals were then transported to the HD facility. Animals were anesthetized with IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed into the biosafety cabinet. Dr. John S. Graham with the aid of Dr. M. Simon then dosed the right dorsal surface with 50 µL of methylene chloride (MC) containing 0 µg HD (Group 1; animal #3-7), 320 µg HD (Group 2, animal #8-12), 160 µg HD (Group 3, animal #13-17) or 80 µg HD (Group 4, animal #18-22). The treatment was added within a cloning ring (See Figure 1A). Beginning at animal #7, the rim of each cloning ring placed on the animal was coated with Krytox (Dupon, GPL203, Lot -G1330) to prevent the noted leaking of solvent from between the animal and the cloning ring. Cloning rings were held in place for 5-minutes to ensure complete evaporation and uptake of the treatment. Each animal was then placed back into its cage. After 24-hours animals were photographed (See Figure 1B) by Dr. Edward Clarkson and then euthanized with by IP injection of pentobarbital (150 mg/kg). Eight mm punch biopsies were taken of the treatment area placed between two thin sponges in a cassette and fixed in formalin. Animal handling, anesthesia and euthanasia were carried out under the direction of Dr. Thomas Zimmerman, D.V.M.

Histology and Immunohistochemistry:

Biopsies were formalin fixed for 24 hours, placed in 70% ethanol and sent to McClain Laboratories, Smithtown, NY for paraffin embedding, staining and histopathology. Using standard procedures 6 µm sections were cut and deparaffinized with xylene and graded alcohols. Histopathology was carried out using H&E [Mayers hematoxylin (PolyScientific, S2697, Lot 04770) and eosin (PolyScientific, S176, Lot 03446) staining] and with PAS [Periodic acid (PolyScientific, S1861-32, Lot 0363), Schiff Reagent (PolyScientific, S272-16, Lot 05334)]; sections were counterstained with hematoxylin to detect nuclei. Immunohistochemistry was carried out with antibody against HMGB1 (ABCAM, AB18256, Lot 389323) according to the manufacturer's instructions using a dilution of 1:50. Antigen was retrieved with a 30 minute, 90^{o}C incubation with DIVA (Biocare, DV2004G1, Lot 111907) after which slides were cooled to room temperature and sections were blocked with Sniper Block (Biocare, BS9662, Lot 060208). Antibody binding was detected using the amplification system developed by Biocare (MACH4 Probe, UP536L, Lot 011008 and MACH4 Polymer, Lot 011008) using Vulcan Fast Red as the chromagen (Biocare, FB803S, Lot 100507). Of the HD treatment group only mouse 13 looked normal upon histological assessment.

Appendix 2.

Table 1. Summary of gross observations and histopathology

Table 2. Image analysis

Table 1: Summary of gross observations and histopathology

Animal #	Treatment	Clinical observation 24hr post-treatment	Epidermal necrosis	Vascular necrosis	Muscle fixation	Inflammatory cell infiltrate	Epidermal nuclear HMGB-1
3*	MC	No lesion observed	Patchy	No	Yes	No	patchy
	none	Normal skin (control)	No	No	No	No	yes
4*	MC	White area in region of MC application	Foci of necrosis	No	Yes	No	patchy
	none	Normal skin (control)	No	No	No	No	yes
5*	MC	No lesion observed	Patchy (50%)	No	Yes	No	patchy
	none	Normal skin (control)	No	No	No	No	yes
6*	MC	No lesion observed	Patchy (>50%)	No	Yes	No	patchy
	none	Normal skin (control)	No	No	No	No	yes
7	MC	White area in region of MC application (well defined)	Patchy	Patchy	Yes	No	patchy
	none	Normal skin (control)	No	No	No	No	yes
8	320 µg HD	Well defined white region with edema beyond the original lesion	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	Few
	none	Normal skin (control)	No	No	No	No	Nuclear
9	320 µg HD	Moderately defined white region with edema beyond the original lesion	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	Few
	none	Normal skin (control)	No	No	No	No	Nuclear
12	320 µg HD	Well defined white region with edema beyond the original lesion	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	Few
	none	Normal skin (control)	No	No	No	No	Nuclear
13	320 µg HD	Poorly demarcated lesion	No	No	No	No	Nuclear
	none	Normal skin (control)	No	No	No	No	Nuclear
14	160 µg	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
15	160µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
16	160µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
17	160µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
18	80 µg	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
19	80 µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
20	80 µg HD	Visible lesion; edema within treatment area (8 mm)	Yes	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
21	80 µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear
22	80 µg HD	Visible lesion; edema within treatment area (8 mm)	Confluent epidermal and follicular necrosis	Yes	Yes	Yes	few
	none	Normal skin (control)	No	No	No	No	Nuclear

*These animals received doses prior to use of Krytox® sealant (DuPont, GPL203 Lot-G1330) at the rim of the cloning ring and so an indeterminate amount of the MC leaked out of the chamber; this is easily seen with the ink dissolved around the cloning ring area (see Figure 1B)

Table 2. Image Analysis

Mouse #	Area	Aspect Ratio	Roundness
3	No obvious lesion (no blanching)		
4	0.7108	1.8090	1.4004
5	No obvious lesion (no blanching)		
6	No obvious lesion (no blanching)		
7	0.3456	1.1695	1.2420
8	0.4387	1.6790	1.3227
9	0.6032	1.1518	1.2357
12	0.4460	1.3490	1.2582
13	0.4335	1.1890	1.2120
14	0.6975	1.5291	1.2975
15	0.7902	1.0070	1.1965
16	0.4023	1.1382	1.2014
17	0.6950	1.4754	1.4311
18	0.4362	1.0993	1.1567
19	0.6331	1.1351	1.2629
20	0.5467	1.0333	1.1998
21	0.5485	1.3420	1.3174
22	0.7414	1.1655	1.2179

Image analysis was carried out on the lesions observed at 24 hours post-treatment. The lack of blanching on animals #3, #5 and #6 prevented analysis. Minimal discoloration on the treatment sites of animal #9, #13, #14, and #16 made these measurements difficult and therefore questionable.

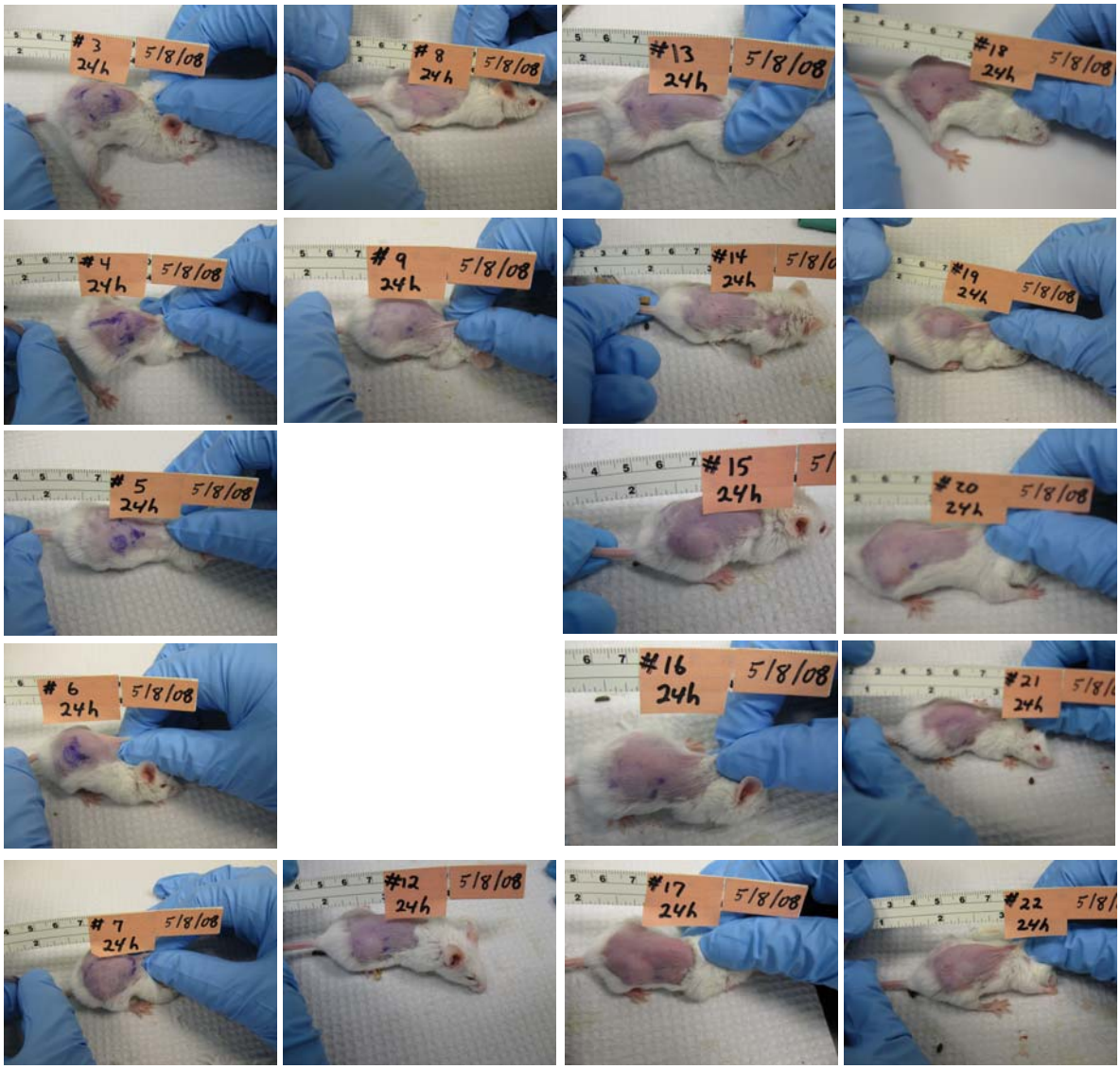
Appendix 3.

Figure 1. Dose ranging

A

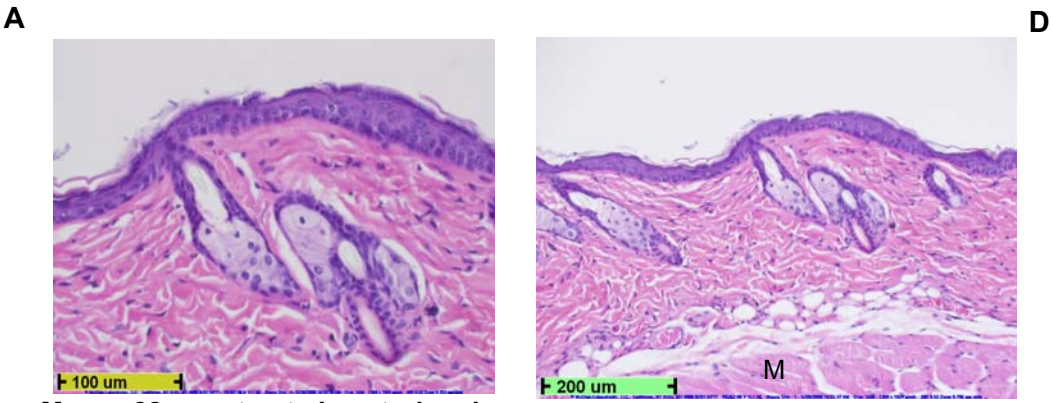
B

Group 1 Methylene chloride	Group 2 HD (320 µg)	Group 3 HD (160 µg)	Group 4 HD (80 µg)

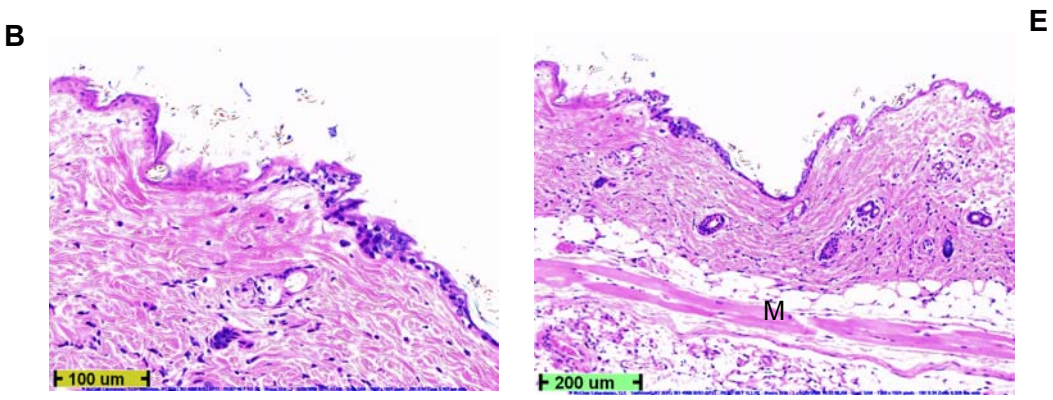


Animals 10 and 11 died during anesthesia recovery.

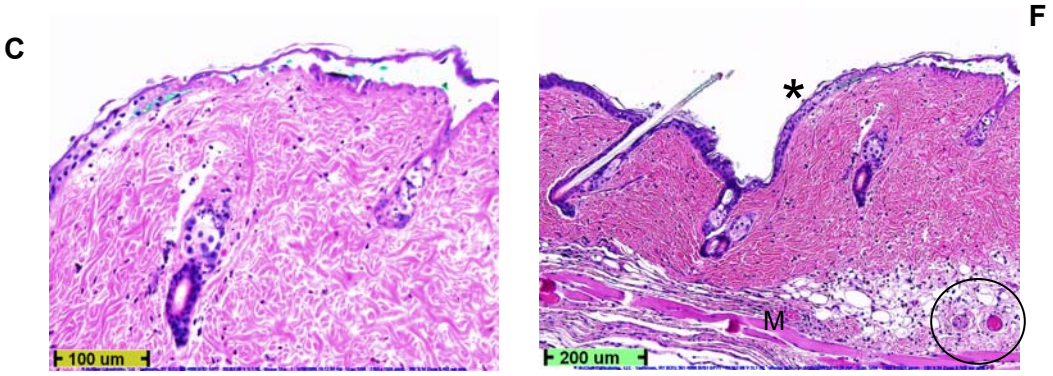
Figure 2. Hematoxylin & Eosin



Mouse 22: non-treated control region



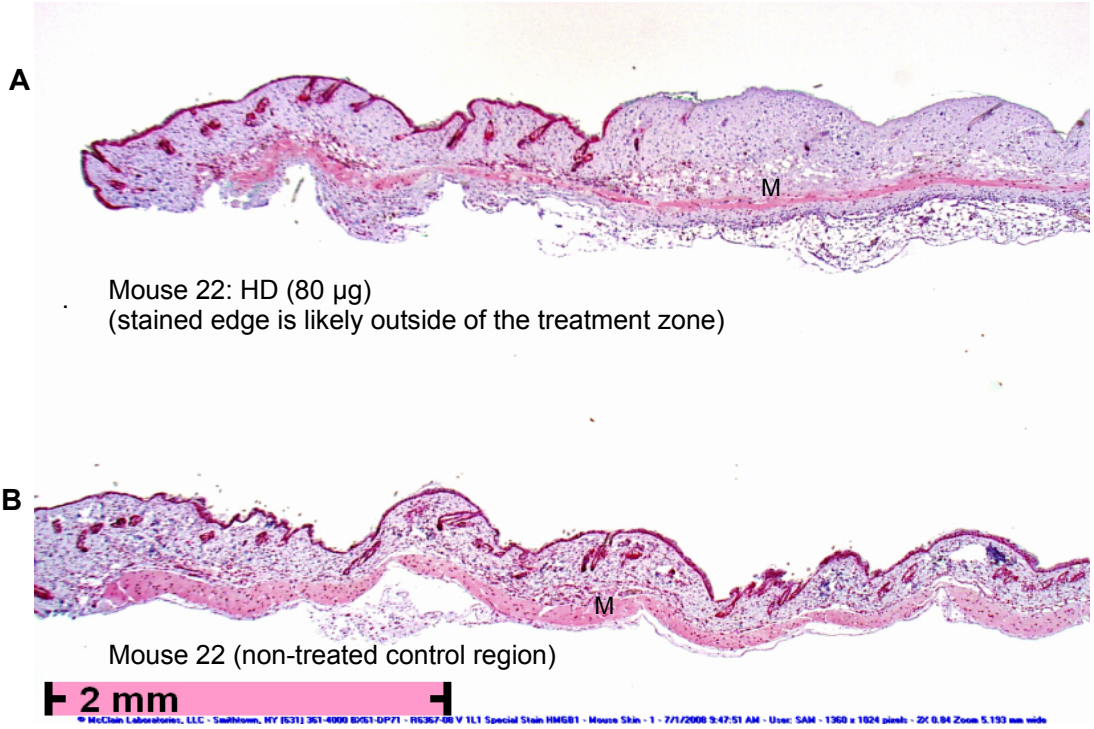
Mouse 6: Methylene chloride

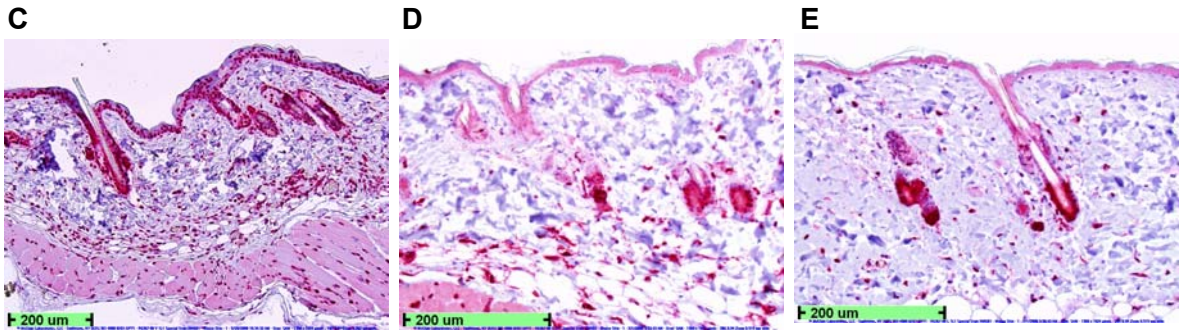


Mouse 22: HD treated (80 µg)

H&E staining of normal mouse tissue (Panel A, D), methylene chloride treated tissue (Panel B,E) and HD treated tissue (Panel C, F) is shown. Epidermal necrosis/apoptosis and muscle (M) injury is apparent in both the vehicle control (methylene chloride) and HD treated skin. Deep dermal injury is limited to the HD treated tissue (circle). Hematoxylin staining at the biopsy edge (*) outside the treatment area is shown for internal comparison.

Figure 3: HMGB1 Staining (Mouse 7, 12, 22)



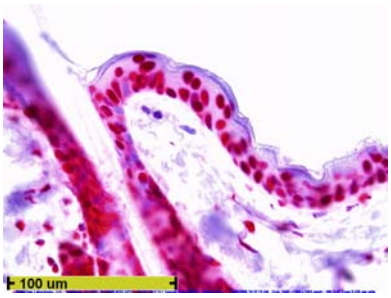


Mouse 22: control

Mouse 7: Methylene chloride

Mouse 12: HD (320 µg)

F

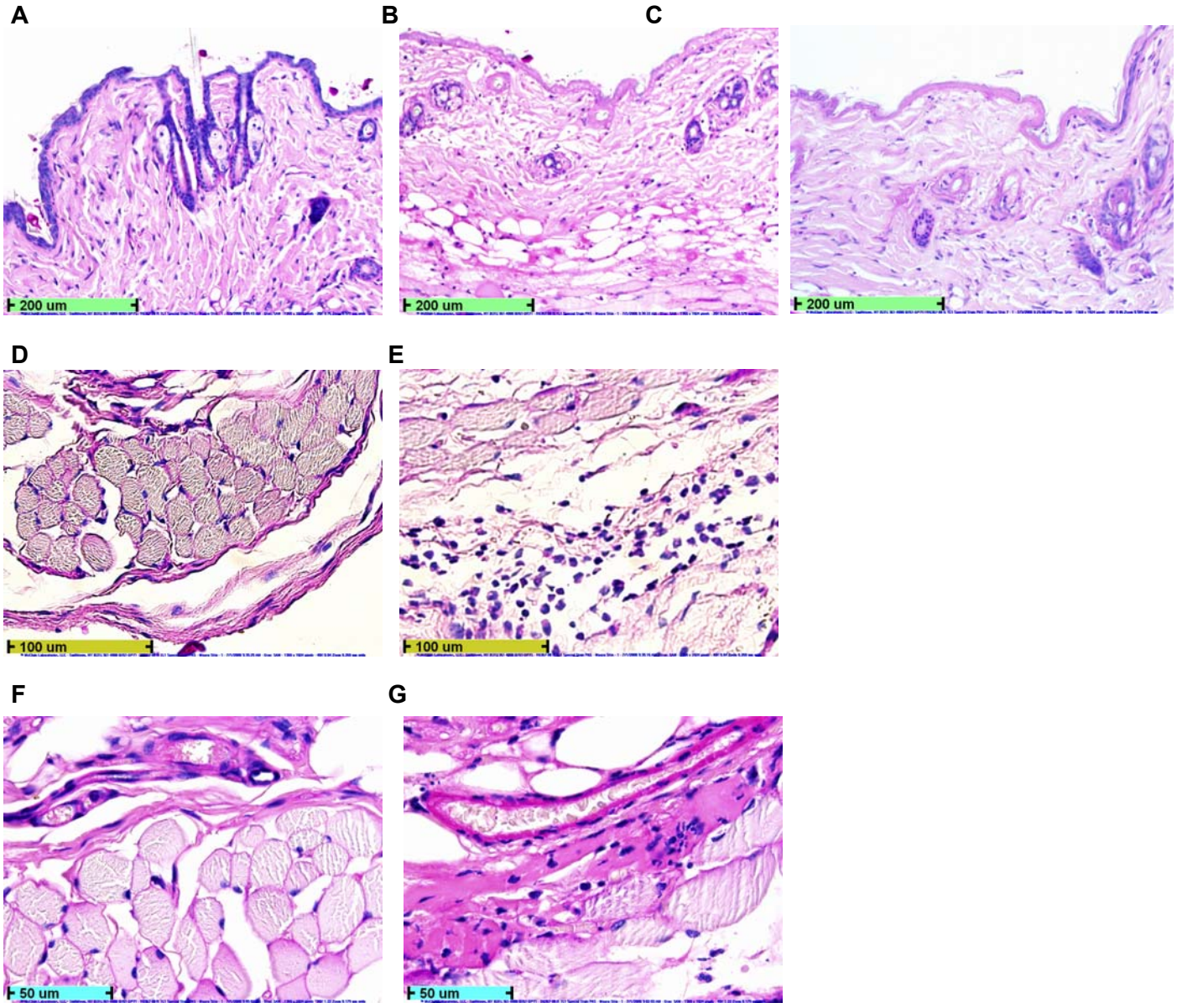


Mouse 22: control

HMGB1 staining of HD treated (Panel A, E), methylene chloride treated (Panel D) and non-treated, normal (Panel B, C, F) mouse skin is shown. Loss on nuclear localization was seen in both the vehicle control (methylene chloride) and HD treated animals.

Figure 4: Hematoxylin/Periodic acid-Schiff (PAS) Staining

Non-treated (Mouse 18) HD (40µg) (Mouse 18) Methylene Chloride (Mouse 7)



The HD treated skin (Panels B, E, G) and control skin (Panels A, D, F) are shown. Comparison of Panels A and B reveals a loss of epidermal nuclear staining; comparison of C and D reveals sub-muscular inflammatory infiltrate (Panel D) limited to the HD treated region and comparison of Panel E and F reveals fibrin leakage (Panel F) limited to the treatment site. Treatment with methylene chloride alone resulted in a reduction of nuclear PAS staining in the epidermis (C). Inflammatory infiltrates were observed only in the HD treatment groups.

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Joel Israel, Director of Research at McClain Laboratories

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